QUANTITATION OF LACTIC ACID IN CAFFEINE-CONTRACTED AND RESTING FROG MUSCLE BY HIGH RESOLUTION NATURAL ABUNDANCE ^{13}C NMR

Donald D. DOYLE and Michael BÁRÁNY*

Department of Biological Chemistry, University of Illinois at the Medical Center, Chicago, IL 60612, USA

Received 24 February 1982

1. Introduction

High resolution natural abundance ^{13}C NMR spectra of intact muscle was reported in [1,2]. The observed resonances could be assigned to carbons located in phospholipids, in proteins and in soluble organic compounds of the muscle.

Out of the major resonances one set at 21, 69 and 183 ppm results from the methyl, hydroxyl and carboxyl carbons of lactic acid, respectively. The hydroxyl resonance at 69 ppm is uncontaminated by other muscle carbon resonances and can be used to directly measure the lactic acid content of muscle. This resonance at the pH of muscle appears ~1 ppm downfield from that of a pD 3.8 lactate placed in a capillary and inserted into the muscle sample. From the known quantity of lactate in the capillary and from the ratio of the integral of the lactate standard signal to that of the muscle lactate signal the lactate content of the muscle can be calculated. With this procedure we followed lactic acid production as a function of time and temperature in caffeine-contracted and resting frog gastrocnemius muscles by natural abundance ^{13}C NMR.

2. Experimental

2.1. *Preparation of muscle and standard lactate*

The gastrocnemius muscle of frog was excised uninjured by cutting the tendon at both ends. Two or three muscles from 2.5–3.5 in frogs or up to as many as 6 muscles from smaller frogs were used. Muscles were soaked in Ringer's solution or in Ringer's solution containing 10 mM caffeine for 10 min at room temperature, blotted and gently packed closely into the bottom of 12 mm sample tubes. A few drops of Ringer's solution was added to the muscle for recording the spectrum to aid in conduction of heat built-up in the muscle by decoupling pulses.

Standard lactate solutions (pD 3.8 and 7.0) were prepared by titrating crystalline lactic acid (Sigma) dissolved in D_2O with NaOH dissolved in D_2O. Lactate solutions at all stages were made <40% to avoid formation of lactic anhydride.

Standard lactate and muscle sample volumes were determined by weighing a column of water in the capillary used and an annulus of water in the NMR tube used, both of equal height. The density of frog gastrocnemius muscle was taken to be 1.06 [3].

2.2. *NMR measurements*

^{13}C NMR spectra of muscle were recorded at 90.546 MHz under spectrometer conditions as in [1]. For quantitation of muscle lactate the capillary inserted in the NMR tube contained 1.68 M lactate (pD 3.8) and 4.4% dioxane in D_2O. Samples were pulsed at 30–50° (7–10 μs) with sufficient delay (1.2–6.4 s) between pulses so that at each experimental temperature all carbons of interest were fully relaxed. The muscle lactate content was calculated from the ratio of the integral of the pD 3.8 lactate standard in the capillary resonating at 68.3 ppm to the integral of the muscle lactate signal at 69.3 ppm. Integrals were measured from the expanded spectral region 66.5–70.5 ppm. Alternatively, in a few cases (fig.2) lactate (pD 7.0) was used as a standard. Since this signal coresonates with that of muscle lactate, the pD 7.0 standard was included in the dioxane-containing capillary, inserted in the muscle sample, and

* To whom correspondence should be addressed

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

recorded at 10°C before or after the muscle lactate itself was recorded with dioxane alone in the capillary.

Spin-lattice relaxation times (T_1) were determined by using a 180°–τ–90° pulse sequence [4]. A total of 1400 scans were collected at each of 10 τ values from 0.1–8.0 s, using caffeine-treated muscles during a 15 h expt. In order to take account of systematic variations in signal strength over the long time of the measurement, 20 scans were collected at each τ value through the cycle of τ values, for 70 cycles. The T_1 values were calculated by the 3 parameter method [5]. Nuclear Overhauser enhancement (NOE) values were determined as in [6].

3. Results

The characteristic resonances of muscle lactate appear at 20.9 ppm (methyl carbon), 69.3 ppm (alcoholic carbon) and 183.2 ppm (carboxylic carbon). If the carboxyl group of lactate is only half dissociated, i.e., pD 3.8, as in the capillary, its characteristic resonances appear slightly upfield from those of muscle lactate. The greatest shift is seen for the carboxylic carbon, 1.7 ppm, the least for the methyl carbon, 0.1 ppm, whereas the alcoholic carbon shifts by 1.0 ppm from 69.3–68.3 ppm.

3.1. *Requirements for quantitation of muscle lactate*

Quantitation of muscle lactate using lactate standards requires knowledge of 3 sets of values: (1) T_1-values; (2) NOE-values of the standard and muscle lactates as a function of temperature [7]; and (3) the strength of a signal emanating from the capillary relative to that of a similar signal emanating from the surrounding volume of the tube. Attenuation of the signal from the capillary by the sample surrounding it could diminish its relative intensity.

The T_1-values for the alcoholic carbon of lactate species considered, pD 3.8 lactate, pH 7.0 lactate, and muscle lactate, increased with increasing temperature. The T_1 values for the pD 3.8 standard ranged from 0.95 s at 10°C–1.88s at 19°C–2.4 s at 29°C. The T_1-values for the muscle lactate were slightly longer but within 20% of the pD 3.8 standard. The T_1-values for the pH 7.0 standard were significantly longer, reaching 3.6 s at 29°C. Because of these variations in T_1-values between muscle lactate and reference standards, pulse length and recycling times were chosen so that full relaxation of the carbons occurred. This

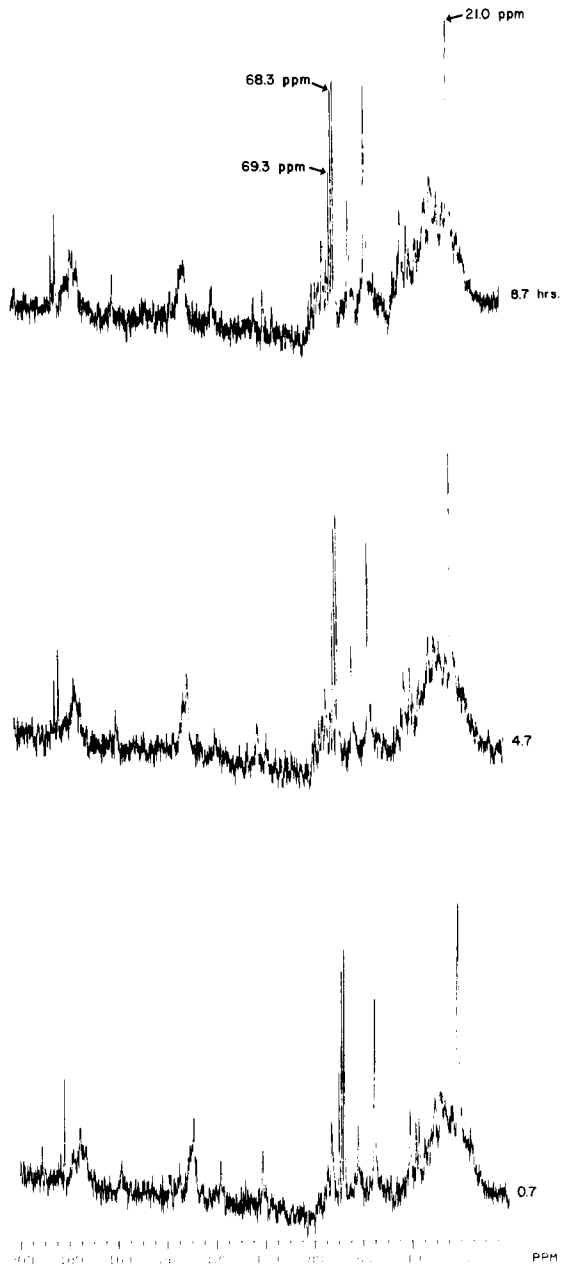

Fig.1. ^{13}C Natural abundance NMR spectra of caffeine-treated frog gastrocnemius muscles recorded at 90.5 MHz. The muscles were treated with 10 mM caffeine in frog Ringer's solution for 10 min, then blotted, and transferred into NMR tubes. The time course of lactate production was measured at 1.33 h intervals (4000 scans) for 9 h at 31°C. The lower, middle and upper spectra were obtained in the first, third, and fifth interval, respectively. A coaxial capillary containing 4.4% dioxane and 1.68 M lactate (pD 3.8) was inserted into the NMR tube to give reference signals at 67.4 and 68.3 ppm, respectively. For other peak assignments see the text and [1].

was checked by measuring the lactate content of resting muscles as a function of time at 28°C (fig.3); pulsing at 35° (7 μs) with 1.2 s recycling time gave values which were colinear with those pulsing at 45° (9 μs) with 6.4 s recycling time.

The NOE value for the alcoholic carbon of the pD 3.8 lactate standard was 2.99 at 18°C, giving the theoretical nuclear Overhauser enhancement factor (NOEF) of 1.988 [7]. At 29°C, the NOEF value was 1.8 for the pD 3.8 lactate. The NOEF values for muscle lactate and the pH 7.0 lactate standard were ~90% of the pD 3.8 lactate standard in all cases. For purposes of quantitation, integrals of muscle lactate were divided by 0.9 when the pD 3.8 standard was used in calibration.

With the pD 3.8 standard in the capillary and the pH 7.0 standard in Ringer's in the surrounding tube, and using the NOE correction with fully relaxed spectra, the amount of lactate at pH 7.0 in the tube could be predicted to ⩽5% from the integrals of the 2 species. Therefore, we made no correction for any possible attenuation of the capillary lactate standard signal when quantitating muscle lactate.

3.2. *The lactate content of caffeine-contracted and resting muscles as a function of time and temperature*

Upon caffeine-treatment frog gastrocnemius muscle contracts and produces a large amount of lactate (fig.1). Quantitation of lactate content of the caffeine-treated muscle as a function of time (fig.1) revealed a slight decrease. Thus, 67, 63 and 59 μmol lactate/g muscle were found after 1, 3.5 and 6 h caffeine-treatment, respectively. Apparently, caffeine-treatment induced maximal lactate production in muscle and the lactate formed was oxidized slowly with aid of the O_2 trapped into the muscle and diffused from the air into the muscle.

Fig.2 depicts lactate formation by a resting frog gastrocnemius as a function of time at 24°C. After 1 h incubation, no 21 or 69 ppm resonance is visible and it takes ~7 h to detect these peaks. However, after 11–13 h the 21.0 and 69.3 ppm peaks are increased demonstrating the accumulation of lactate in the resting muscle.

The lactate concentration of muscle is plotted as a function of time in fig.3 for resting muscle at 22, 24 28 and 43°C and caffeine-treated muscle at 24, 31 and 32.5°C. The high temperature dependence of lactate production in frog muscle is apparent. Thus, the experimental points delineating lactate production at 24°C are clearly above the points at 22°. The rate of lactate production is 2.0 μmol . h^{-1} . g $muscle^{-1}$ at

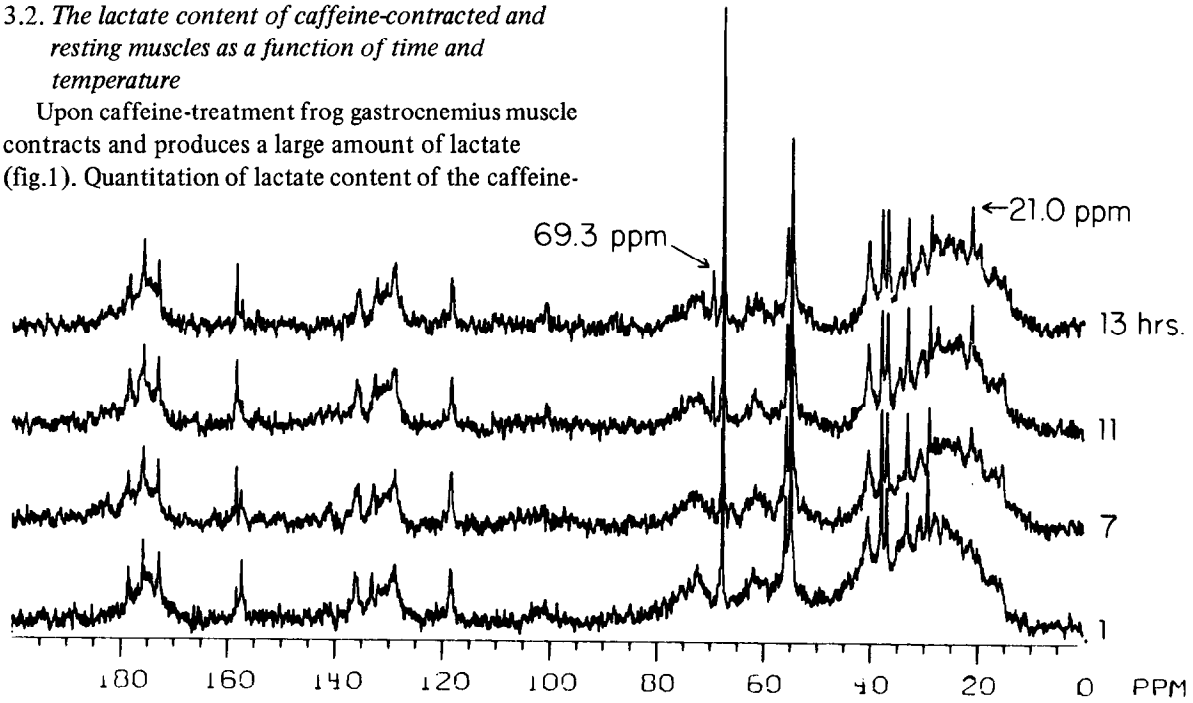

Fig.2. ^{13}C Natural abundance NMR spectra demonstrating the accumulation of lactate in frog resting gastrocnemius at 24°C. Muscles were excised and loaded into an NMR tube on ice and equilibrated in the instrument for 10 min to the running temperature. Spectra of 6000 scans each (2 h) were collected. The peaks at 21.0 and 69.3 ppm are due to lactate methyl and hydroxyl carbons. The large peak at 67.4 ppm is from the dioxane external standard.

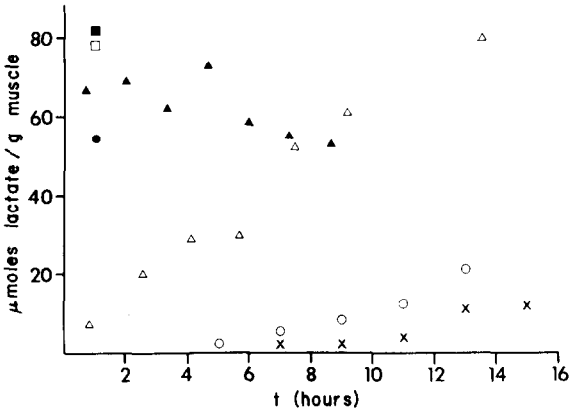

Fig.3. The build-up of lactate in resting and caffeine-treated frog gastrocnemius muscle as a function of time: resting muscle at 22°C (×), 24°C (○), 28°C (△) and 43°C (□); caffeine-treated muscle at 24°C (●), 31°C (▲) and 32.5°C (■). The temperature of the muscles was controlled as in [1] and it was measured by a hand thermometer after the scanning was completed.

24°C and 6.5 μmol . h^{-1} . g^{-1} at 28°C. The lactate content is maximal, ~82 μmol/g, at 43°C in resting muscle at the earliest time of determination. The lactate content of caffeine-treated muscle is significantly greater than that of the corresponding resting muscle at room temperature. At a given temperature, the lactate content of caffeine-treated muscle appears to be maximal at the first time interval measured and it decreases after 5 h.

4. Discussion

Quantitation of lactic acid by ^{13}C NMR agrees with its chemical determination, reported in the literature. Thus, the lactate content of caffeine-contracted muscles at 31–33°C, 72–82 μmol/g, as determined by ^{13}C NMR, is in agreement with the chemically determined value of 79 μmol/g in frog muscles treated with 2,4-dinitrofluorobenzene for 24 h [8]. In frog muscles stimulated for 15 min lactate accumulation amounted to 43 μmol/g [9]. The low lactate concentration in resting frog muscle estimated by ^{13}C NMR agrees with the chemical determinations in [10], 1.4 μmol lactate/g unstimulated frog muscle. Furthermore, 1.2 μmol lactate was found/g unstimulated rat muscle [11]. In [12] the change in chemical shift of P_i, due to the protons generated from lactic acid in fatigued muscle, was used for estimation of lactic acid by ^{31}P NMR. They measured ~30 μmol lactate in frog muscles stimulated at various frequencies for a prolonged time.

Direct determination of lactic acid by the non-destructive natural abundance ^{13}C NMR technique has the potential to measure the extent of glycolysis, under physiological conditions, in functionally different skeletal, heart and smooth muscles. It seems likely that this technique can also be applied to quantitate glycolysis in other intact tissues, e.g., liver or brain.

We monitored the lactate content of human muscles obtained as residual samples from orthopedic surgery. The lactate concentration of diseased muscles varied greatly, apparently reflecting a difference in glycogen breakdown. Thus, high resolution natural abundance ^{13}C NMR may be useful to detect the disturbance of carbohydrate metabolism in muscle.

Acknowledgement

This work was supported in part by the University of Illinois NSF Regional Instrumentation Facility, grant NSF CHE 79-16100, and by the Muscular Dystrophy Association. We gratefully acknowledge the enthusiastic help of David G. VanderVelde.

References

[1] Doyle, D. D., Chalovich, J. M. and Bárány, M. (1981) FEBS Lett. 131, 147–150.
[2] Bárány, M., Doyle, D. D., Graff, G., Westler, W. M. and Markley, J. L. (1982) J. Biol. Chem. in press.
[3] Wilkie, D. R. (1953) J. Physiol. 119, 369–375.
[4] Freeman, R. and Hill, H. D. W. (1970) J. Chem. Phys. 53, 4103–4105.
[5] Canet, D., Levy, G. C. and Peat, I. R. (1975) J. Magn. Res. 18, 199–204.
[6] Levy, G. C. and Peat, I. R. (1975) J. Magn. Res. 18, 500–521.
[7] Levy, G. C., Lichter, R. L. and Nelson, G. L. (1980) Carbon-13 nuclear magnetic resonance spectroscopy, pp. 36–41, Wiley, New York.
[8] Infante, A. A. and Davies, R. E. (1965) J. Biol. Chem. 240, 3996–4001.
[9] Nassar-Gentina, V., Passonneau, J. V. and Rapoport, S. I. (1981) Am. J. Physiol. 241, C160–C166.
[10] Nassar-Gentina, V., Passonneau, J. V., Vergara, J. L. and Rapoport, S. I. (1978) J. Gen. Physiol. 72, 593–606.
[11] Sahlin, K., Edstrom, L., Sjoholm, H. and Hultman, E. (1981) Am. J. Physiol. 240, C121–C126.
[12] Dawson, M. J., Gadian, D. G. and Wilkie, D. R. (1978) Nature 274, 861–866.